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Astringency reduction in red wine by whey proteins ☆

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ABSTRACT

Whey is a by-product of cheese manufacturing and therefore investigating new applications of whey proteins will contribute towards the valorisation of whey and hence waste reduction. This study shows for the first time a detailed comparison of the effectiveness of gelatin and β-lactoglobulin (β-LG) as fining agents. Gelatin was more reactive than whey proteins to tannic acid as shown by both the astringency method (with ovalbumin as a precipitant) and the tannins determination method (with methylcellulose as a precipitant). The two proteins showed similar selectivity for polyphenols but β-LG did not remove as much catechin. The fining agent was removed completely or to a trace level after centrifugation followed by filtration which minimises its potential allergenicity. In addition, improved understanding of protein–tannin interactions was obtained by fluorescence, size measurement and isothermal titration calorimetry (ITC). Overall this study demonstrates that whey proteins have the potential of reducing astringency in red wine and can find a place in enology.

1. Introduction

Red wine is a beverage that is rich in phenolic compounds, mainly tannins, but also other smaller molecular weight phenolics which have demonstrated to have many health benefits due to several biological activities, such as antioxidant, cancer preventing and anti-inflammatory activity (Middleton, Kandaswami, & Theoharides, 2000). Tannins are high molecular weight (over 500 Da) polyphenols, which have the ability of precipitating with gelatin and other proteins in solution. These proteins are in general rich in proline. The tannins occurring in wine are responsible for the undesired sensorial properties, especially astringency. Astringency is a rough or drying mouth-feel that causes a puckering sensation, associated with interactions between polyphenols from wine and certain proteins from saliva. Mainly the proline rich proteins (PRPs) from saliva are the ones responsible for this sensation (Mehansho, Butler, & Carlson, 1987). According to Charlton et al. (2002), and as described by Dinnella, Recchia, Fia, Bertuccioli, and Monteleone (2009), binding and precipitation of polyphenols by PRPs involves a multi-step mechanism. At first, reversible hydrophobically-driven binding of the polyphenol to the protein takes place to give a soluble complex. Then, more polyphenol is added and cross-linking of peptides occurs, the complex becomes insoluble, and, finally, further aggregation (phase separation) of the insoluble complexes occurs. The intensity of this sensation depends on the polyphenolic composition and concentration in wine, as well as on the palate of the individual tasting it. Some proteins, such as casein, gelatin, egg albumin, and isinglass are known to interact with phenolic compounds in a similar way to saliva proteins (Guerrero, Smith, & Bindon, 2013), improving clarity, sensory characteristics and aging capacity of wines. The model for polyphenol–protein interactions described above is also valid to explain interactions between tannins and these proteins.

When a protein treatment is applied to wine, care must be taken for the molecules used to be selective. Otherwise, most of the beneficial properties from wine phenols could be lost in the process. Moreover, it is important to reduce astringency to a limit where it does not result in extensive precipitation of polyphenols. It is desired that some mild astringent sensation remains, for a winemaker should have the ability to modulate astringency by adjusting the balance accordingly. For all these reasons, the study of the protein–phenolic interactions is very important.

Whey obtained from cheese manufacture has low commercial value and represents an alternative for the obtention of proteins that can interact with phenolics. The addition of milk to tea has shown to result in complexation of milk proteins and tea catechins without impairing the bioavailability of the catechins and improving its sensorial properties (Kanakis et al., 2011; Ye, Fan, Xu, & Liang, 2013). Whey proteins and in particular β-LG (the major whey protein) has also shown to interact with polyphenols in tea (Kanakis et al., 2011) and to complex with particular polyphenols

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(von Staszewski et al., 2012) however, it has never been applied to the reduction of red wine astringency. β-LG is a small globular protein of 18,350 Da which possess a hydrophobic pocket that shows particular affinity for hydrophobic molecules. β-LG has some technological advantages for its application as a fining agent: inexpensive, food grade and non-toxic material, capable of solubilising and protecting hydrophobic biologically active molecules in aqueous media whilst retaining the sensory properties.

Tannin–protein interactions have been investigated by a wide range of physicochemical techniques (Frazier, Papadopoulou, & Green, 2006; McRae, Falconer, & Kennedy, 2010). In this work we used isothermal titration calorimetry (ITC) to gain better understanding of the affinity of β-LG and gelatin for tannic acid, model tannin from wine. ITC provides information not only on thermodynamic parameters and the strengths of the tannin–protein interactions but also on the stoichiometry of the resulting complex. The conformation of the protein is of critical importance as random coil proteins have a higher interaction with tannins than globular proteins. Therefore, the comparison of β-LG with a widely used random coiled protein, gelatin, is included.

The aim of this work was to assess the efficiency of reduction of astringency of whey proteins particularly, β-LG and a mixture of β-LG and caseinomacropeptides (CMP) and to compare them against gelatin. The whey samples were produced by a combination of ion exchange and microfiltration following a method developed in our group (Welderufael, Gibson, & Jauregi, 2012). Astringency was assessed by an analytical method developed by Llaudy et al. (2004) which relies on the precipitation of tannins by ovalbumin. They found good correlation between this analytical method and the sensory evaluation of astringency in a range of wines. Further investigation of the tannin–protein interactions was also carried out by fluorescence, dynamic light scattering (particle size) and ITC.

2. Materials and methods

2.1. Materials

All Chemicals used were of analytical grade. Bovine β-lactoglobulin (β-LG), bovine serum albumin (BSA), bicinchoninic acid solution (BCA), copper sulphate solution, DEAE Sepharose®, rennet, ovalbumin, tannic acid, tartaric acid, potassium monophosphate, potassium diphosphate, sodium hydroxide, sodium chloride, hydrochloric acid (32.5%), trifluoroacetic acid (TFA), methanol, ethanol, methylcellulose, ammonium sulphate, acetonitrile, catechin, epicatechin, gallic acid and type B gelatin from bovine skin were purchased from Sigma–Aldrich (Dorset, UK). Flat sheet microfiltration membranes (0.45 μm), syringe driven PVDF Filters (0.45 μm) were sourced from Millipore Corporation (Bedford, UK). Protease N 'Amano' Enzyme from Bacillus subtilis was purchased from Amano Enzyme Inc. (Nagoya, Japan). Pasteurised Skimmed milk and Merlot red wine, La Chasse Merlot (2012), from France (13% alcohol) were purchased from local stores.

2.2. Preparation of sweet whey from skimmed milk and purification of peptides

Skimmed milk was heated to 35 °C in a water bath. Commercial rennet was added at a concentration of 0.3 mL per litre of milk with gentle stirring for 2 min. Incubation took place for one hour at that temperature and then the casein coagulum was cut in small squares to allow the remaining lactoserum to drain out of it. Incubation was extended for 20 additional minutes and then the coagulum was scooped and filtered to drain the most of the serum with the aid of vacuum. The whey was centrifuged at 3200 RCF and filtered with 0.45 μm syringe driven filter to remove the last of the left over casein curds.

The sweet whey was fractionated to obtain a β-LG rich fraction and a fraction containing CMP and β-LG following method developed in our group (Fig. S1) based on a combination of adsorption and microfiltration (Welderufael et al., 2012). Nitrocellulose microfiltration membrane was cut and placed into the 150 mL ultrafiltration magnetically stirred Amicon cell. To begin the purification process, 100 mL of whey (pH 6.4) and 10 mL of resin were added to the cell and stirred for 10 min. The mixture was filtered through the membrane, with the aid of positive pressure of air. The mixture was micro-filtered to separate the non-adsorbed proteins from the adsorbed proteins. The resin was washed with 10 mM potassium phosphate buffer at pH 6.5. Adsorbed proteins (β-LG and CMP) were desorbed and eluted with known volume of elution buffer, 10 mM potassium phosphate buffer at pH 4.5. For an enriched β-LG fraction without CMP, a hydrolysis step was introduced while proteins were adsorbed to the resin (S1). Hydrolysis started after re-solubilising the adsorbed proteins with a pH 7, 10 mM potassium phosphate buffer, at 45 °C. Then, protease 'N'Amano enzyme was added to the mixture. After 2 h, hydrolysed CMP were microfiltered and finally, the non-hydrolysed protein remaining, β-LG, was desorbed and eluted with 10 mM potassium phosphate buffer at pH 4.5 containing 0.5 M NaCl. Total protein content was analysed by BCA method, and HPLC was used for qualitative analysis of proteins.

2.3. Total protein content

Total proteins were quantified according to the bicinchoninic acid assay (BCA). Briefly, 100 μl of standard or sample was mixed with 2 mL of the BCA working reagent (copper sulphate solution: BCA solution at a ratio of 1:50). The mixture was allowed to stand at 37 °C for 30 min, and then allowed to cool at RT for 5 min. Finally, absorbance was read for each sample/standard, at 562 nm within 8 min with water as a blank. Bovine serum albumin was used as a standard for protein quantification.

2.4. Whey protein analysis by HPLC

The major whey proteins, (β-LG, alpha-lactalbumin, BSA) could be identified using RP-HPLC, with a method adapted from Thoma, Krause, and Kulozik (2006). Samples of known total protein content were filtered through a 0.45 μm PVDF filter and analysed in a Dionex HPLC with a P680 pump, ASI-100 automated sample injector, thermostated column compartment TCC100, PDA-100 photodiode array detector with C-18 column (250 × 4.6 mm). A gradient of solvent A (0.1% TFA in water) and solvent B (0.08% TFA in ACN) was utilised in the following way: B 0–45% in 0–60 min, B 45–70% in 60–65 min, 70% B in 65–75 min, and finally 0% B in 75–90 min. The column temperature was set at 40 °C. The flow rate was 0.8 mL/min, injection volume was 50 μl and the absorbance of the samples was monitored at 214 and 280 nm.

2.5. Particle size measurement

Dynamic light scattering was used to monitor the interaction between tannic acid and protein, and to determine the size of the β-LG nanoparticles, using a Zetasizer Nano S (Malvern instrument, Malvern, UK), equipped with a 4 mW He-Ne laser (633 nm). Before measurements, samples were filtered (0.23 μm) and diluted (1:10 in the corresponding buffer of preparation). In another experiment with the same equipment disposition, the size of complex formed between tannic acid (0.2–1.2 mg/mL) and β-LG (0.1 and 0.5 mg/mL) was monitored. Measurements were